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Complete genome sequence of *Bacillus pumilus* NWMCC0302, a strain for degrading bovine blood

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Abstract

Background Directly discharging livestock and poultry slaughter blood without proper treatment can cause severe ecological damage. Exploring the use of microorganisms to break down waste blood into smaller molecules such as peptides and amino acids, as well as investigating the possibility of transforming these small molecules into water-soluble fertilizers containing amino acids, holds significant research value in the comprehensive utilization of livestock and poultry blood.

Results In this study, a single strain of *Bacillus pumilus* NWMCC0302, which has effectively degraded bovine blood, was isolated from abattoir blood sludge using casein agar plates and Columbia blood agar plates. The degradation test was carried out using bovine raw blood as a nitrogen source in the medium, and the results showed that the strain degraded 53.83% of bovine blood under optimal degradation conditions. The whole genome sequencing of *Bacillus pumilus* NWMCC0302 was conducted using the second-generation DNBSEQ platform and the third-generation PacBio platform, employing high-throughput sequencing technology. The size of the strain's entire genome was determined to be 3 868 814 bp with a G-C content of 41.63%. The total gene length accounted for 88.98% of the genome length at 3 442 341 bp and encoded 4 113 genes. The strain contained 79 tRNAs, 24 rRNAs, 7 sRNAs, and 296 repetitive sequences. The gene data obtained from sequencing were also functionally annotated using the COG, KEGG, and VFDB databases. In the COG database, 310 genes were involved in amino acid transport and metabolism, including 10 catabolic proteins related to COGs. In the KEGG database, were 201 genes involved in amino acid metabolism pathways, including 8 genes in nitrogen metabolism pathways and 2 genes in oxidative pathways. The VFDB database contains two lysostaphin genes and one serine protease-hydrolyzed ClpP gene.

Conclusions In summary, *Bacillus pumilus* NWMCC0302 was screened for its efficient ability to degrade bovine blood. Additionally, the genetic information of *Bacillus pumilus* NWMCC0302 was revealed at the genetic level, providing a feasible experimental method for applying this strain to the degradation of blood from slaughtered livestock and poultry. Moreover, it is a potential functional strain for producing amino acid-containing water-soluble fertilisers.

Keywords *Bacillus pumilus*, Bovine blood degradation, Genome sequence, Function annotation

Introduction

China possesses a diverse range of animal species and a substantial quantity of animal blood, making it one of the world's wealthiest reservoirs. This is particularly evident in the blood obtained from the slaughter of domesticated livestock and poultry. In 2022, China's National Bureau of Statistics reported the slaughter of 48.4 million bovines,

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336.24 million sheep, 699.95 million pigs, and 1,614 thousand poultry. The weights of pigs, bovine, sheep, and poultry at the time of slaughter were determined to be 100 kg, 500 kg, 25 kg, and 2.5 kg, respectively (Bennett and Williams 1995; Van den Broeke et al. 2020). It is projected that in 2022, the total blood volume from the slaughter of livestock and poultry in China will reach 7.6869 million tons, underscoring the substantial scale of animal slaughter on a global level. The blood of livestock and poultry is rich in bioactive substances and nutrients, with a dry matter content ranging from 17.00% to 21.00% of the total blood (Banks and Zhang 2012). It contains abundant haemoglobin and plasma protein while having low-fat content (0.15%—0.20%), making it a significant source of protein (Bah et al. 2013). Despite this, only a small portion of the blood from livestock and poultry slaughter in China is consumed or processed into by-products due to cultural beliefs, living customs, and slaughtering methods. The utilization rate does not exceed 10%, resulting in the majority being discharged as waste. However, appropriate management and utilization of livestock and poultry slaughter blood can yield substantial economic benefits, reduce environmental pollution, and enhance living conditions, which are crucial for safeguarding human health. Nevertheless, an objective assessment of the potential uses of livestock and poultry blood is essential due to its complex spatial structure, strong odour, as well as diverse components such as haemoglobin and fibrin (Schechter 2008; Salzano et al. 2015). The challenge lies in fully realizing the inherent potential during the application and promotion process, which significantly hinders the development and utilization of these resources. Currently, there is no efficient treatment or comprehensive recycling approach available for managing discarded blood, which leads to resource wastage and environmental pollution.

Currently, the blood of domestically and internationally slaughtered livestock and poultry is primarily utilized for the production of high-protein blood feed (Aladetohun and Sogbesan 2013), plasma protein powder (Amer et al. 2022), blood protein peptides (In et al. 2002), and biologically active substances (Bah et al. 2016). A small portion is also used to manufacture edible animal products such as blood tofu, blood meal, and plasma powder. The traditional methods of processing livestock and poultry slaughter blood include direct drying or spray drying fresh blood into blood powder, as well as processing livestock and poultry blood into small molecular fragments through cook-drying, puffing, microbial fermentation, and acid–base hydrolysis. However, these methods have high investment costs, low utilization rates, complex processes, and the destruction of various nutrients like peptides and amino acids during treatment. These problems pose biological safety concerns and limit the widespread application of livestock and poultry blood (Terrumun and Oliver 2015). Therefore, choosing a new and effective method to treat blood during livestock and poultry slaughter can aid in resource recycling, environmental protection, increased utilization rates, and enhancing the added value of livestock and poultry blood. This is an essential matter concerning the safe treatment and comprehensive utilization of animal and poultry blood resources.

The research has shown that microorganisms and proteases effectively break down proteins into hydrolysates, such as small molecule peptides and amino acids (Zheng et al. 2018; da Silva Bambirra Alves et al. 2021). These degraded products can create water-soluble fertilisers rich in amino acids, have low processing costs, simple degradation conditions, and low pollution (Gonzalez-Fernandez et al. 2015). The blood of livestock and poultry is rich in protein, amino acids, iron, potassium, sodium, and other nutrients. Adding a certain amount of blood protein powder to the feed for livestock and poultry can increase the protein content of the feed and accelerate their growth rate and weight gain (Amer et al. 2022). Through strain selection and process improvement, fermented blood meal has a higher amino acid content and better palatability than directly dried or boiled blood meal (Cheng et al. 2024). In Germany, Britain, Japan, and other countries, livestock and poultry feed will be made from blood micro-ecology. Various strains of *Aspergillus oryzae* (Zheng et al. 2014), *yeast* (Zhang et al. 2014), and *Bacillus subtilis* (Wang et al. 2015a) with strong protease-producing capacity are used to ferment the blood of livestock and poultry in order to improve the utilisation of blood meal protein. Wang (2007) utilised *Aspergillus oryzae* to degrade blood meal as animal feed, thereby enhancing the palatability and digestibility of fermentation by-products. Cheng (2022) utilised *Aspergillus oryzae*, *yeasts*, and *Bacillus* to degrade pig blood meal and produce a high-protein feed abundant in vitamin D, niacin, and trace elements. Relevant studies have shown that feeding micro-ecological blood meal effectively inhibits chicken dysentery by 94.50 per cent and piglet dysentery by 89.60 per cent (Zhou et al. 2007). Livestock blood contains various trace elements and a wide range of amino acids required by plants, but the level of free amino acids in livestock blood is low. Jeon (2013) used degraded pig's blood, prepared as a liquefied fertiliser rich in amino acids, to enhance grasses' nitrogen, magnesium, and chlorophyll content and promote their growth. The microbial composting degradation using livestock and poultry slaughter wastes to prepare organic fertilisers for the cultivation of soybean and maise (Nunes et al. 2015), tomato (Roy et al. 2016), pepper and amaranth (Bhunia

et al. 2021) significantly improved the activity and structure of the soil and regulated the balance of microflora in the soil, and significantly improved crop yield and quality.

The blood from livestock and poultry can be effectively utilized and developed to maximize its value. This is a promising research area for degrading and comprehensively utilizing waste blood. In order to investigate the mechanism of microbial degradation of livestock and poultry blood, we employed bacteria and protease enzymes to break down the waste material. Through this biological process, the large protein molecules in the blood were broken down into smaller molecular products like polypeptides and amino acids. Afterwards, these smaller molecular products were converted into water-soluble fertilizers that contain amino acids.

This study reports the isolation of a strain of *Bacillus pumilus* from abattoir sewage sludge that efficiently degrades bovine blood. The whole genome of *Bacillus pumilus* was sequenced using high-throughput sequencing technology. Based on the genome components, gene function annotation, and analysis of genes related to protein degradation, further exploration was conducted on the mechanism of protein degradation and biological properties of *Bacillus pumilus*. Initially thought to provide a foundation for further research on functional genomics and degradation properties, the genes related to protein degradation and metabolic pathways in the strain are discussed. This text serves as a basis for future research into the functional genomics and degradation characteristics of *Bacillus pumilus* to achieve environmentally sound treatment and complete reuse of waste protein resources from livestock and poultry slaughter wastes. This research provides new insights for promoting green and sustainable development in handling livestock and poultry slaughter wastes.

Materials and methods

Bacteria strains and culture conditions

Bacillus pumilus NWMCC0302 was isolated from sewage and the sludge of an abattoir in Maqu County, Gansu Province, China. The strain's isolation site was in the southwestern region of Gannan Tibetan Autonomous Prefecture, Gansu Province, northwestern China. It is situated at a longitude of 103°14′35.41″E, a latitude of 35°36′16.20″N, and an altitude of approximately 1918 m. The collected samples were serially diluted in sterile saline, coated separately on casein medium plates, and placed in a constant temperature incubator with 55% humidity and a temperature of 37 °C for 48 h. The single colony with the largest transparent hydrolysis circle was selected and inoculated onto Columbia blood agar plates for further incubation. After another 48 h, the single colony with the largest haemolysed circle was chosen again and further inoculated onto Columbia blood agar plates. The culture was continued at 37 °C until single colonies with unique characteristics were obtained through purification and culture. The strain is preserved at the Joint National Laboratory of China and Malaysia, Northwest Minzu University. The individual strains were identified through morphological, physiological, and biochemical experiments and 16S rRNA gene sequence analysis. The bacterium was cultured twice in Luria Bertani medium before being added to the blood fermentation medium with a 1% inoculum.

Bovine blood protein degradation

The blood degradation test used a 250 mL conical flask as the incubation vessel. In this test, 20 mL of yak blood, 21.31 g/L of wheat bran, and sterile water were added to the flask as the blood degradation medium. The total volume of the medium was adjusted to 100 mL. The samples were then fermented and cultured for 96 h at an inoculation amount of bacterial species set at 2.0%, a temperature of 38.1 ℃, and a rotational speed of 180 r/min. After fermentation, the free amino nitrogen content in the samples was determined using the double indicator formaldehyde titration method. In contrast, the Kjeldahl nitrogen determination method determined the total nitrogen content. The degradation rate of blood proteins was calculated based on the ratio between the free amino nitrogen content and total nitrogen content in each sample.

Determination of free amino acid content in degradation products

The blood degradation products were freeze-dried into powder form using a lyophiliser to remove the proteins. Then, 5-sulfosalicylic acid with a mass fraction of 5% was added, and the mixture was centrifuged at 1200 r/min for 15 min. After centrifugation, 10 μL of the supernatant was extracted. The free amino acids in the degradation products were detected using a fully automated amino acid analyser with a 4.6×60 mm sulfonic acid cationic resin column. The separation column temperature was set to 50 ℃, and the reaction column temperature was set to 135 ℃.

Genome sequencing and assembly

The bacterial isolates were cultured in Luria Bertani liquid medium at 37 ℃ and 180 r/min for 12 h until they reached logarithmic growth. The bacterial liquid was collected in a sterile centrifuge tube and centrifuged at 4000 r/min for 5 min at room temperature. The supernatant was discarded, and the bacterial cells were washed with sterile water and centrifuged again for 5 min. The bacterial cells were then transferred to another sterile

centrifuge tube, frozen with liquid nitrogen, preserved in dry ice, and sent to Novogene for whole-genome sequencing using the PacBio Sequel, a third-generation sequencer. K-mer analysis was utilised to predict genome size, heterozygosity, and repetitive sequence content. The optimal assembly result, Clean Data, was achieved after multiple adjustments. Subreads were extracted from the Polymerase Reads, and adapter sequences were filtered out. The Subreads were mixed and corrected using Proofread software to obtain highly reliable Corrected Reads. Finally, the optimal assembly results were selected using Falcon software. The Falcon software was used to select the optimal assembly result, followed by single base correction using GATK software to obtain highly reliable assembly sequences. The assembly results were then subjected to sequence loop judgement, genome sequence and plasmid sequence differentiation, and the valid data were analysed by combining various software.

Component, gene annotation and gene function analysis

The software Glimmer was utilised to predict gene start sites and coding regions. Multiple software tools were employed to analyse genomic components, including repetitive sequences, coding genes, non-coding RNAs, prophages, gene islands, and CRISPR. The gene set of the sequenced strain was compared with COG, KEGG, CAZy, VFDB, and CARD databases using Diamond software. Gene function annotation and categorisation were then performed to determine the function of the genes and their related descriptive information. The study compared the predictions to sequences of haemoglobin-degrading bacteria available in NCBI. This resulted in the identification of genes and proteins associated with haemoglobin degradation. Possible degradation pathways were constructed using the KEGG database.

Results

Isolation, screening, morphology, and identification of bacterial strains

Bacillus pumilus NWMCC0302 was screened by measuring the hydrolysis zone of the strain on casein medium plates and the hemolysis zone on Columbia blood agar plates. After being cultured on casein medium plates at 37 °C for 24 h, *Bacillus pumilus* NWMCC0302 formed milky white colonies with round shapes, raised and smooth surfaces, neat edges, and an opaque appearance. The colony diameters were approximately 1.0–1.4 mm, while the transparent hydrolysis circle had a diameter of about 4.5 mm (Fig. 1A). After incubating at a constant temperature of 37 °C on Columbia blood agar plates for 24 h, the colonies exhibited a greyish-white colour. They had rounded, raised, smooth surfaces with neatly defined edges. They were also glossy and sticky in appearance. The diameter of the colonies was approximately 2.7–4.2 mm, while the size of the hemolyzed zone was about 10.0–12.0 mm in diameter (Fig. 1B). The rod-shaped bacteria observed under the oil immersion lens appeared bluntly rounded at both ends and arranged singly or in pairs with bud cells. Further analysis through biochemical tests indicated that these bacteria belonged to an aerobic Gram-positive strain. CLSI antimicrobial drug susceptibility test results show that *Bacillus pumilus* strain NWMCC0302 showed low susceptibility to ceftazidime. Analysis of the 16S rRNA gene sequence revealed a homology rate of 99.93% with *Bacillus pumilus* strain ATCC7061.

Experimental analysis of bovine blood degradation

The study investigated the degradation of bovine blood by *Bacillus pumilus* NWMCC0302 under various culture conditions. The results indicated that the strain had a degradation effect of 17.34% on bovine blood,

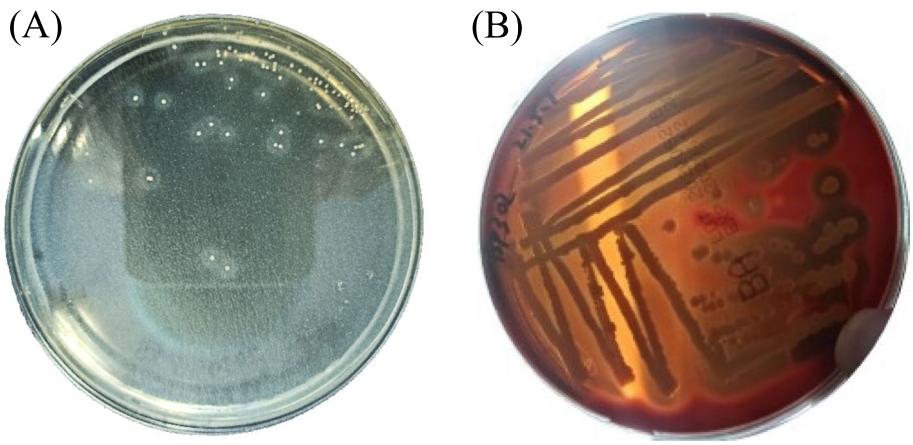


Fig. 1 Morphological characteristics of *Bacillus pumilus* NWMCC0302 colonies on casein plates (**A**) and blood agar plates (**B**)

which was not optimal. Therefore, it is necessary to explore the effects of different fermentation times, carbon sources, inorganic salts, temperatures, initial pH, and inoculation amounts on the degradation of bovine blood. This will improve the strain's ability to degrade bovine blood. Based on the analysis of the optimised results, the medium used 20 mL of bovine raw blood as the nitrogen source. The key factors affecting bovine blood degradation were identified as the initial pH, bran concentration, inoculum amount, and degradation temperature. Under the initial fermentation conditions of pH 7.39, bran concentration of 21.31 g/L, strain inoculum of 2.0%, degradation temperature of 38.1 ℃, and degradation time of 96 h, the degradation effect on bovine blood was found to be 53.83%. This is 3.10 times higher than the effect before optimization.

Analysis of free amino acid content in degradation products

Many microbial strains produce extracellular proteases during fermentation that break down large proteins into small polypeptide and amino acid-based hydrolysates. These strains can be grown under aerobic and anaerobic conditions to produce enzymes capable of breaking down blood proteins. The degradation products of bovine blood proteins degraded by *Bacillus pumilus* contained 24 free amino acids (refer to Table 1). The product contains 14 free amino acids, including cysteine, aspartic acid, and phenylalanine, which promote plant growth. Additionally, leucine and valine, the most abundant amino acids in the product, promote seed germination and seedling development. If the degradation products are used to create amino acid water-soluble fertilisers, they can increase seed germination rates and improve crop flavour. Furthermore, amino acid degradation products contain numerous peptides and other small molecules that can enhance crop growth. According to the national standard for the preparation of bioorganic fertilisers (NY 1429–2010), amino acid-containing water-soluble fertilisers should have a free amino acid content of at least 10.0% (w/v), and the degradation products should have a total free amino acid content of 56.21 mg/mL. The degradation products of this strain on bovine blood and China's bio-organic fertiliser preparation still have deficiencies. These can be adjusted by formulating the bacterial agent and adjusting the ratio of the material and liquid to improve the free amino acid content of the degradation products of bovine blood. Alternatively, the degradation products of bovine blood can be compounded to achieve the standards of amino acid water-soluble fertiliser production and utilisation.

Table 1 Analysis of degradation products of bovine blood protein by *Bacillus pumilus*

Types	Times /min	Area	Concentration (nmol/mL)	CalcMol (ng/mL)
P-Ser	1.920	60,433	85.69	15,861.45
Tau	2.607	89,668	144.26	18,061.61
Asp	9.300	10,077,520	14,877.73	1,980,225.34
Thr	13.800	1,898,076	2710.24	322,789.60
Ser	15.207	712,279	976.67	102,647.74
$AspNH_2$	17.007	8499	16.58	2190.02
Glu	18.933	9,164,853	13,603.52	2,001,077.43
$GluNH_2$	20.893	16,390	22.29	3255.88
Pro	30.820	728,847	3031.06	348,874.49
Gly	33.153	7,433,007	10,003.05	750,929.23
Ala	35.080	70,441,310	97,136.63	8,653,902.59
Val	41.173	60,698,794	83,652.85	9,795,749.00
Cys	43.507	15,668	20.05	4818.18
Met	44.740	9,918,302	13,860.79	2,068,030.50
Ile	47.280	1,758,565	2441.67	320,347.20
Leu	48.500	64,746,495	92,933.45	12,192,869.02
Tyr	50.887	7,543,241	11,413.53	2,068,132.32
Phe	53.513	28,495,676	42,511.38	7,022,880.08
g-ABA	60.760	93,594	125.56	12,945.20
Trp	63.440	3,688,488	6948.41	1,418,866.24
Orn	72.287	1,920,841	2124.42	280,847.82
Lys	74.100	37,378,349	46,187.80	6,752,657.00
His	76.853	297,470	392.73	60,951.35
Arg	86.260	41,197	58.28	10,152.62
		317,227,562	445,278.64	56,209,061.91

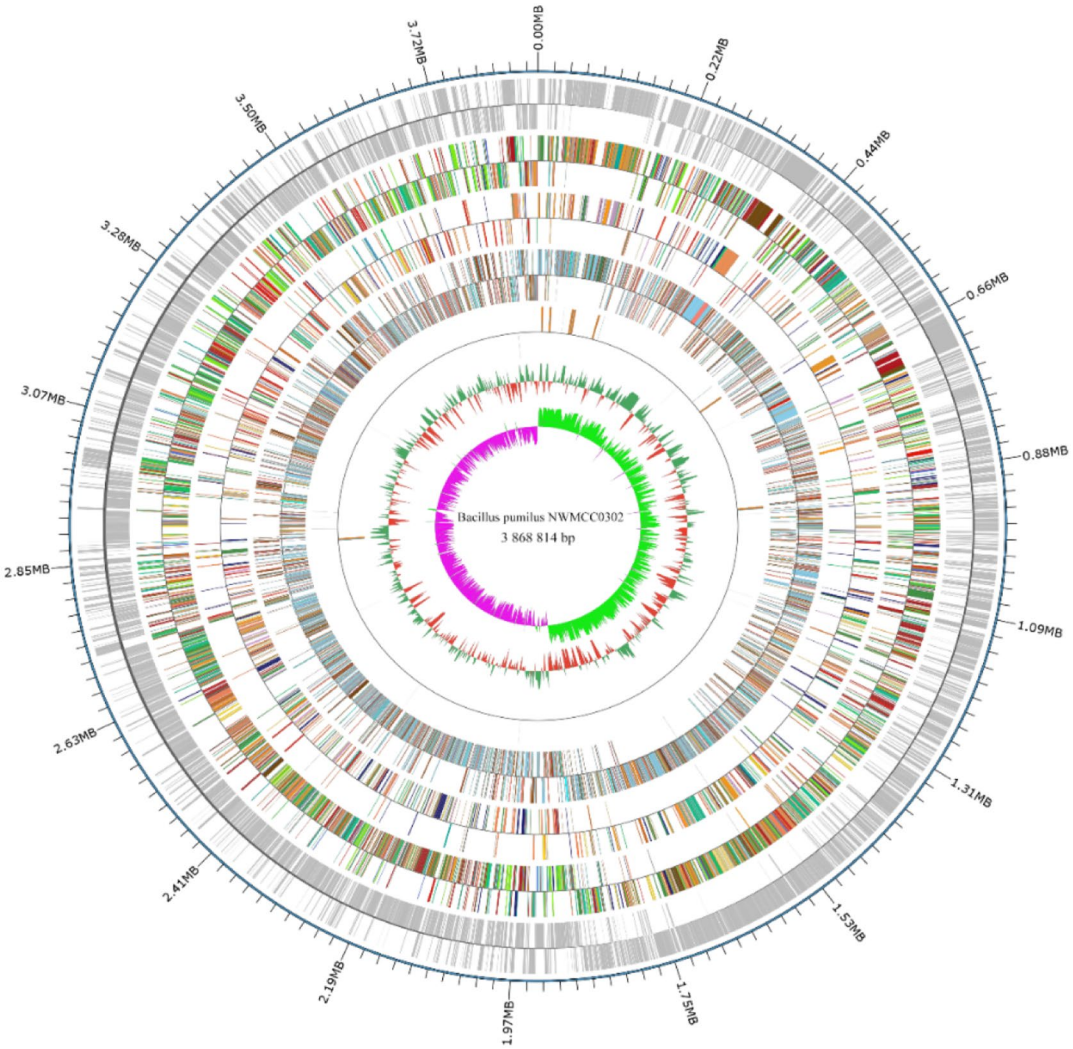
Genome component

The genome assembly and optimization of *Bacillus pumilus* NWMCC0302 resulted in a total genome size of 3,868,814 bp and a G-C content of 41.63%. This is 0.43% and 0.23% higher than that of *Bacillus pumilus* ATCC 7061 (Ojaghi et al. 2018) and *Bacillus pumilus* MTCC B6033 (Villanueva et al. 2014), respectively. This strain exhibits a higher proportion of G-C base pairs than the two standard strains, resulting in increased DNA double helix thermal stability. The whole genome sequencing of *Bacillus pumilus* NWMCC0302 resulted in the annotation of 4113 coding genes using various databases. Among these, 3836, 2895, 2680, 3987, 2680, and 143 genes were annotated using KEGG, COG, GO, NR, Pfam, and CAZy databases, respectively. The total gene length was 3 442 341 bp, accounting for 88.98% of the total genome length. The gene annotation rate was 100%, and a total of 479, 198, 3, 45, and 2866 functional genes were identified. The genome sequence contained a total of 110 ncRNAs, comprising 79 tRNAs, 24 rRNAs, and 7 sRNAs. Additionally, 296 repetitive sequences were

identified, including 4 CRISPRs and 6 prophages. The repetitive sequences consisted of 195 tandem repeats and 140 minisatellite DNAs, but no microsatellite DNAs were found. The genome-wide genome mapping results were combined with the prediction of the coding genes. The whole genome of *Bacillus pumilus* NWMCC0302 was mapped using Circos software based on the genome-associated information (Fig. 2).

COG annotation

The protein sequences of *Bacillus pumilus* NWMCC0302 were compared to the COG database to annotate the equivalent genes to their corresponding homologous sequence COG clusters. The functional annotation results of the genes are presented in Fig. 3. A total of 2895 protein-coding genes were annotated to the COG database, and 3269 coding genes were specifically functionally



The outermost circle is the genomic sequence position coordinates, and from the outer to the inner circle are (1) coding gene, (2) COG functional classification, (3) KEGG functional classification, (4) GO functional classification, (5) ncRNA, (6) genomic G+C content, and (7) genomic GC-skew value

Fig. 2 Whole genome circle map of Bacillus pumilus NWMCC0302. The outermost circle is the genomic sequence position coordinates, and from the outer to the inner circle are (1) coding gene, (2) COG functional classification, (3) KEGG functional classification, (4) GO functional classification, (5) ncRNA, (6) genomic G + C content, and (7) genomic GC-skew value

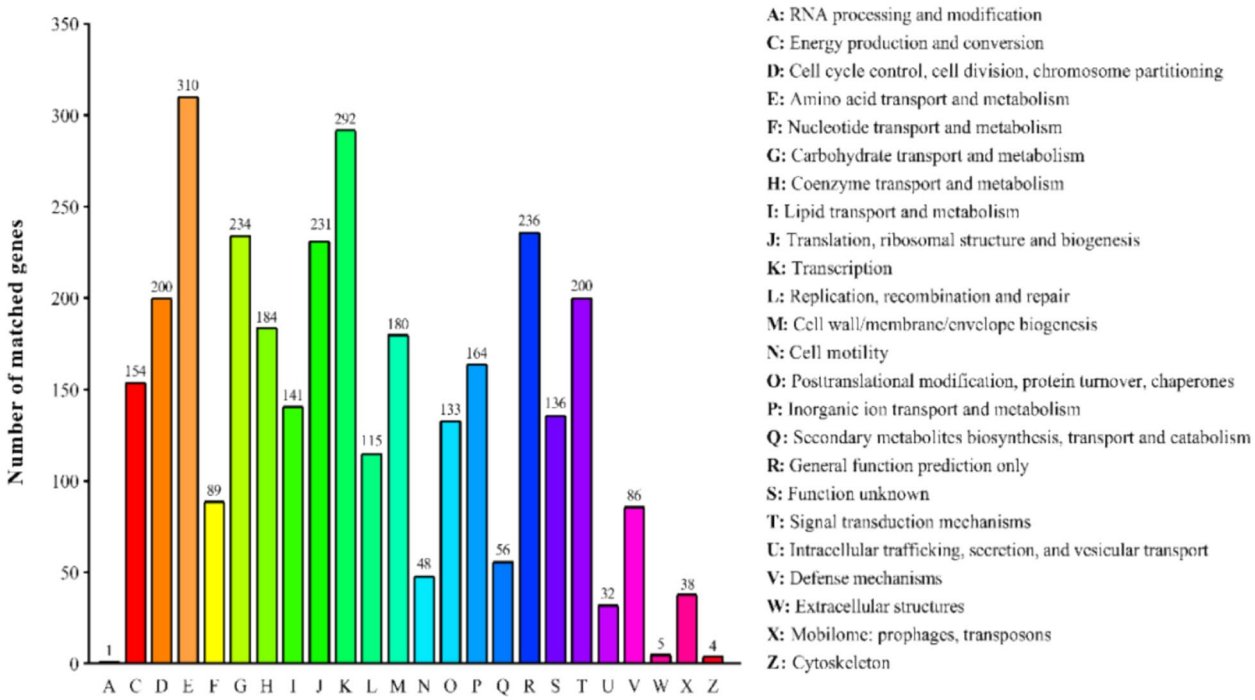


Fig.3 Functional annotation of the genomic COG database of Bacillus pumilus NWMCC0302

classified into 24 categories. The genes identified in this study were primarily involved in carbohydrate transporter metabolism (234 genes, 7.16%), transcription (292 genes, 8.93%), translation, ribosome structure and biogenesis (231 genes, 7.07%), signal transduction mechanisms (200 genes, 6.12%), and general function prediction (236 genes, 7.22%). Additionally, 136 genes were found to have unidentified functions that require further exploration. In COG annotation, *Bacillus pumilus* NWMCC0302 was found to primarily perform carbohydrate metabolism and amino acid transport metabolism. Based on the COG function classification of *Bacillus pumilus* NWMCC0302, the most abundant function was amino acid transport and metabolism, with 310 genes annotated, accounting for 9.48% of the total number of COG-annotated genes. These genes include COG0833 (amino acid permease), COG2066 (glutaminase), COG0531 (serine protease), COG1164 (oligoendopeptidase), COG3104 (dipeptide/tripeptide permease), and COG0527 (aspartate permease). COG1027 (aspartate deaminase), COG3616 (serine deaminase), COG2362 (aminopeptidase), and COG0006 (proline aminopeptidase) are relevant COGs for the catabolism of proteins in amino acid transport and metabolic features. Additionally, the database predicts many putative intracellular proteases in *Bacillus pumilus* NWMCC0302, indicating that this strain has a strong potential for protein degradation.

KEGG annotation

The functional annotation information of *Bacillus pumilus* NWMCC0302 genes was combined, and the metabolic pathway of this strain was comparatively analysed using the KEGG database. The annotation results are shown in Fig. 4, with 3640 genes annotated into six secondary functional categories. Out of the 203 pathways, the metabolism-related pathway had the highest number of genes, with 1614 genes representing 44.34% of the total annotated genes. The amino acid metabolism pathway had 201 genes, the nitrogen metabolism pathway had 8 genes, the carbohydrate metabolism pathway had 225 genes, and the amino acid biosynthesis pathway had 128 genes. The protein decomposition and metabolism involve various amino acid metabolic pathways, including the TCA cycle (25 genes), glycolysis/glyconeogenesis (42 genes), alanine, aspartate and glutamate metabolism (31 genes), glycine, serine and threonine metabolism (35 genes), cysteine and methionine metabolism (41 genes), valine, leucine and isoleucine degradation (22 genes), arginine and proline metabolism (23 genes), and phenylalanine metabolism (5 genes). The nitrogen metabolism pathways of *Bacillus pumilus* NWMCC0302 were compared in the KEGG database. The results showed that eight genes related to reduction pathways and two genes related to oxidation pathways were up-regulated in *Bacillus pumilus* NWMCC0302, confirming its strong nitrogen utilization ability.

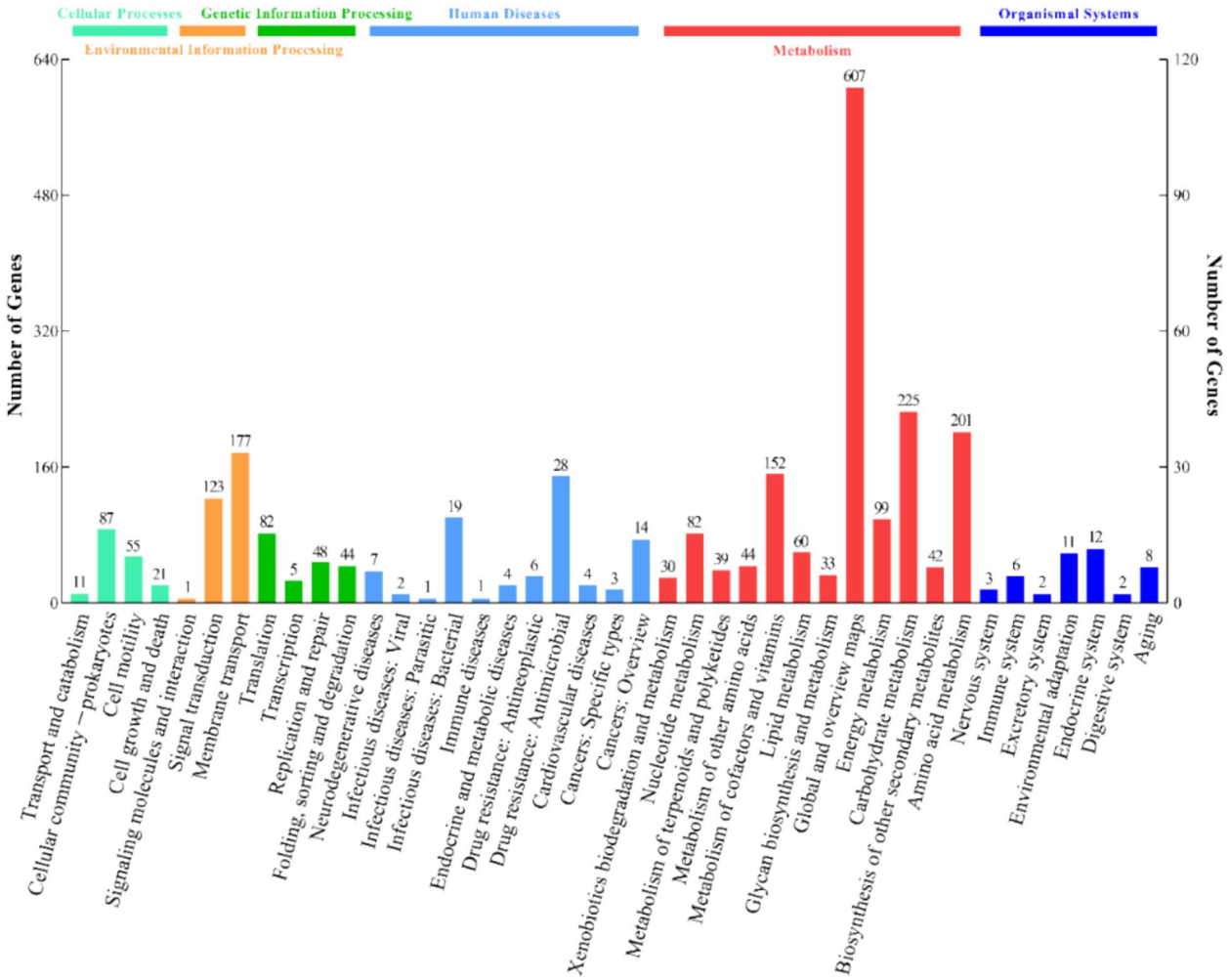


Fig. 4 Functional annotation of the genomic KEGG database of Bacillus pumilus NWMCC0302

Carbohydrate-active enzymes (CAZymes)

The carbohydrate-active enzymes are responsible for the biosynthesis and breakdown of carbohydrates and glycoconjugates. They play a crucial role in various metabolic pathways and are essential for carbohydrate metabolism in microorganisms. Therefore, their significance should not be underestimated. The function of the *Bacillus pumilus* NWMCC0302 strain gene was analysed using the CAZy database. The study found 143 genes that encode protein domains associated with the CAZy family, which accounts for 3.48% of all encoded genes. These genes include 58 for glycoside hydrolases, 29 for glycosyltransferases, 18 for carbohydrate esterases, 2 for polysaccharide lyases, 4 for oxidoreductases, and 39 for carbohydrate-binding modules. The CAZy database of *Bacillus pumilus* NWMCC0302 has identified several protein genes that encode various enzymes, including chitosanases, chitinases, β-glucosidases, acetyl xylan esterases, cellulases, moss enzymes, and others. These enzymes play vital roles in the cleavage and metabolism of macromolecules, such as cellulose. Cellulose is a complex enzyme that comprises three hydrolases: endoglucanase, exoglucanase, and cellulase. These hydrolases hydrolyse cellulose into glucose and polysaccharides. Hemicellulose is broken down into small-molecule polysaccharides or monosaccharides by enzymes such as xylanase, glucanase, and mannanase. The enzyme system, consisting of two enzymes, enhances the ability of *Bacillus pumilus* NWMCC0302 to utilise various organic matter as a nutrient source, thereby improving its survival. Additionally, these enzymes significantly improve the composition and structure of the soil, providing good nutrients for plant growth. This suggests excellent probiotic capabilities.

VFDB, CARD annotation

Studying the virulence factors and resistance genes of *Bacillus pumilus* NWMCC0302 can enhance our

understanding of the strain. Metabolites produced by the strain, such as toxins and invasiveness, aid in host tissue and cell invasion, immune response evasion, and nutrient acquisition for proliferation and growth. The genome sequence of *Bacillus pumilus* NWMCC0302 was analysed in the VFDB database, and 198 virulence factor genes were identified. These genes are mainly involved in bacterial adhesion and colonisation, host immune system evasion, and bacterial toxins production. *Bacillus pumilus* NWMCC0302 possesses virulence factors that can facilitate invasive processes, including the inhibition of complement-mediated phagocytosis, stimulation of inflammation, and bacterial adhesion to host target cells. The strain possessed two cytolysin genes (VFG045470) that can lyse erythrocytes, polymorphonuclear leukocytes, macrophages, and a wide range of Gram-positive bacteria. Additionally, a serine protease gene (VFG000077) involved in protein hydrolysis and a haemoglobin permease gene (VFG034354) were predicted, further confirming the strain's ability to degrade blood proteins. The strain possessed two cytolysin genes (VFG045470) that can lyse erythrocytes, polymorphonuclear leukocytes, macrophages, and a wide range of Gram-positive bacteria. Additionally, a serine protease gene (VFG000077) involved in protein hydrolysis and a haemoglobin permease gene (VFG034354) were predicted, further confirming the strain's ability to degrade blood proteins. In conclusion, the genes annotated in VFDB that were obtained from *Bacillus pumilus* NWMCC0302 are not true virulence genes. Instead, they are regulatory genes that are important in regulating biological processes. Additionally, they are not resistant to most classes of antibiotics. This suggests that the degraded blood products are safe and harmless for use in the production of amino acid-containing aqueous fertilisers, and in biocontrol.

Discussion

The safe disposal of blood waste from livestock and poultry slaughtering is crucial to current agricultural solid waste management and the circular economy (Mian et al. 2017). Large-scale and intensive farming produces significant waste blood during the slaughtering process due to the increasing demand for meat products. This blood waste can seriously pollute the ecological environment if not effectively treated and utilised. However, despite the urgent need for an effective treatment and comprehensive recycling method, the current situation results in the wastage of resources and environmental pollution caused by used blood. In this study, a strain of *Bacillus pumilus* NWMCC0302 was isolated from degrading bovine blood, which can efficiently break down large molecular proteins into small polypeptides and amino acids. Since the critical degradation mechanism of this bacterium is complex to uncover using traditional methods, whole genome sequencing of this strain was performed to explore its genetic resources.

In the COG database, 3269 genes were annotated. The 48% of all genes were related to proteolysis. *Bacillus pumilus* NWMCC0302 was found to have the highest number of genes related to amino acid transport and metabolism, as well as carbohydrate transport and metabolism. A total of 3,640 genes were annotated in the KEGG database, with the highest number of genes (1,614) being in the metabolism-related pathway. In the KEGG database, *Bacillus pumilus* NWMCC0302 had the highest number of genes annotated to amino acid metabolic pathways, with carbohydrate metabolism and amino acid metabolism having the most annotated genes. Among these, 201 genes were involved in the amino acid metabolism pathway, and 8 genes were involved in the nitrogen metabolism pathway. The annotation results from these two databases were consistent. The presence of extracellular protease genes in the genome of *Bacillus pumilus* NWMCC0302 has also been annotated in the KEGG database, indicating its ability to degrade insoluble proteins in the environment into various oligopeptides and amino acids as a means of utilizing nitrogen-containing compounds for growth. Genes encoding serine proteases *Epr* and *Vpr*, neutral proteases, rod peptidases, and the related protease *WprA* have also been identified in this strain's genome, highlighting its efficient protein degradation capability in blood. The results of the previous study showed that the protease produced by *Bacillus pumilus* NWMCC0302 has neutral and serine characteristics. The strain's metabolic production of serine protease indicated that both the *AprX* gene and the *NprE* gene are important genes affecting extracellular protease production in *Bacillus pumilus*. The related studies have shown that, in addition to the genes encoding the protease *Apr* and the neutral metalloproteinase *Mpr*, *Apx* is also an important gene that affects the extracellular protease-producing capacity of bacteria belonging to the genus *Bacillus* (Toymentseva et al 2017), and these findings are consistent with those of our present study.

In the CAZy database annotation results, *Bacillus pumilus* NWMCC0302 contains genes coding for permitted α-amylase, β-glucosidase, β-xylan endonuclease, endoglucanase, G-type lysozyme, and chitinase-related enzymes. The related studies have shown that *Bacillus pumilus* produces cellulases such as β-glucosidase, chitinase, and β-xylan endonuclease.(Shali et al 2017; Aditi et al. 2018) The cellulose can be rapidly absorbed by the animal body by decomposing microorganisms and transformed into an energy-supplying substance during growth and development. The carbohydrate-degrading

enzymes produced by *Bacillus pumilus* play an indispensable role in this process. G-type lysozyme exhibits a broad-spectrum antimicrobial effect and has a wide range of applications in antiviral and antitumor activities. Additionally, *Bacillus pumilus* NWMCC0302 was found to have genes encoding cell wall degrading enzymes such as chitinase, suggesting that the strain may inhibit the life activities of pathogenic microorganisms by breaking down their cell walls.

In the VFDB database, *Bacillus pumilus* NWMCC0302 was also annotated with 198 virulence factor genes, mainly involved in bacterial adhesion and colonization, evasion of the host immune defence system, and production of bacterial toxins. Bacilysin is a class of peptide antimicrobial substances that exhibit potent antibacterial activity against both fungi and bacteria (Nannan et al. 2021), which provides further justification for using strain *Bacillus pumilus* NWMCC0302 in biocontrol. Two cytolysin genes capable of lysing erythrocytes, a *ClpP* serine protease gene for protein hydrolysis and a haem permease gene were annotated in the VFDB database, confirming the genes associated with the degradation of blood proteins by this strain.

The genes related to amino acid transport and metabolism, as well as carbohydrate transport and metabolism, accounted for the highest percentage of genes in the genome of *Bacillus pumilus* NWMCC0302, as annotated by the COG, KEGG, and CAZy databases. It was hypothesized that the presence of these metabolizing enzymes was an intrinsic factor in *Bacillus pumilus* NWMCC0302 ability to degrade blood proteins in livestock and poultry efficiently.

In this study, *Bacillus pumilus* NWMCC0302 demonstrated superior haemoglobin utilisation compared to existing strains that degrade haemoglobin in livestock. Fermentation experiments on blood meal were conducted by Dawei Yao using *Bacillus pumilus* under optimal conditions, including an initial pH of 9.0, an inoculum volume of 4%, and an incubation temperature of 50 °C. The haemoglobin degradation effect was 85% after 36 h (Yao et al. 2012). At the optimum temperature of 33.8 °C, pH 7.0, inoculum content of 7.2×10^7 CFU/mL and 250 r/min, *Bacillus subtilis* degraded haemoglobin by 62.05% over a period of 69.36 h (Wang et al. 2015a, b). In a similar study, Asha (2018) found that after 24 h of fermentation of porcine haemoglobin by *Bacillus cereus*, the degradation rate was 53.29%, and the soluble protein in the degradation product was 1.05%. *Bacillus pumilus* can break down large protein molecules found in livestock and poultry blood into smaller peptide and amino acid products. This makes it a potential candidate for microbiological methods of degrading such blood.

Bacillus pumillus is a rod-shaped bacterium that belongs to the *Bacillus genus* in the diatom family. Its cells are arranged singly or in chains, are motile, and grow aerobically or partially anaerobically. They stain positively with Gram's stain (Bergey et al. 1994). *Bacillus pumilus* is primarily found in soil or colonises plant roots. It is a valuable microbial resource due to its tolerance and ability to suppress soil-borne fungal diseases and nematodes. *Bacillus pumilus* secretes metabolites, including lipase (Zhang et al. 2009), alkaline serine keratinase (Jagadeesan et al. 2020), cellulase (Kundu et al. 2018), xylanase (Garg et al. 2011), and chitinase with strong activity. These metabolites have probiotic functions, such as antiviral properties and improving crop quality. It has strong antimicrobial activity against plant pathogens, can maintain the micro-ecological balance of plants and promote plant growth, and has a wide range of applications in plant probiotic effects and biodegradation (Lanna-Filho et al. 2017; Hao et al. 2019). *Bacillus pumilus* is also able to promote digestion and absorption, regulate the micro-ecological balance of animal intestinal tract, enhance animal immune function and production performance and other beneficial effects (Haldar et al. 2016). For example, three compounds with antimicrobial activity were isolated from the concentrated fermentation broth of *Bacillus pumilus* with strong inhibitory effects against *Staphylococcus aureus*, *Micrococcus luteus*, *Pasteurella multocida*, *Salmonella* porcine, and *Escherichia coli* in chickens (Chu et al. 2019). In addition, *Bacillus pumilus* can also decompose carbohydrates (Moriya et al. 2007), proteins (Reddy et al. 2017), lipids (Saranya et al. 2014), and other substances, which can maximize the use of industrial waste, animal and plant waste and leather products. Therefore, *Bacillus pumilus* has a variety of biological functional characteristics and needs to be further studied and exploited.

Many microbial strains produce extracellular proteases during fermentation that can break down large proteins into small peptides and amino acid hydrolysates, but single strains and proteases have limited ability to degrade livestock blood (Álvarez et al. 2012; Wang et al. 2016). The incomplete use of blood by strains during hydrolysis is due to the complex spatial structure of haemoglobin and fibronectin in livestock blood. This affects strains' growth and metabolism process, preventing them from completely decomposing blood proteins into small molecules such as polypeptides and amino acids, ultimately impacting the degradation effect (Kim et al. 2022). Microbial strains' ability to degrade blood proteins depends on the amount of their proteases produced and enzyme activity. The degradation mechanism of microbial strains on blood proteins is more complex than the protease hydrolysis process. It involves protease hydrolysis of

blood protein substrates and the use of blood protein substrates for the growth and reproduction of strains. Therefore, the culture conditions can affect the growth of strains. Currently, research on livestock and poultry blood uses focuses on the degradation and utilization of haemoglobin and plasma proteins. However, there has been less emphasis on the degradation of raw blood from livestock and poultry slaughtering, which results in the underutilization of blood resources. A synergistic bacterial-enzymatic approach has been discovered to achieve complete degradation of livestock and poultry slaughter blood, leading to effective environmental treatment and resource utilization of slaughter waste.

Conclusion

In this study, a strain of *Bacillus pumilus* NWMCC0302 with the ability to degrade bovine blood was isolated from abattoir sewer sludge, and the results showed a degradation rate of 53.83% for bovine blood. The whole genome of *Bacillus pumilus* NWMCC0302 was sequenced using high-throughput sequencing technology, and it had a total size of 3 868 814 bp with a G-C content of 41.63%, encoding 4 113 genes. Based on bioinformatics software, functional annotation of this strain's whole genome has resulted in 10 catabolic proteins associated with COGs in the COG database. According to the KEGG database, eight genes were involved in the nitrogen metabolic pathway, and two genes were involved in the oxidative pathway. Two cytolysin genes and one serine protease *ClpP* gene for protein hydrolysis were annotated in the VFDB database. The genetic and genomic functional properties of *Bacillus pumilus* NWMCC0302 were revealed at a gene level, which further confirmed its strong ability to utilize nitrogen and degrade proteins, providing a reference for its application in methodical treatment and recycling of slaughter waste blood resources.

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Authors' contributions
XJ: methodology; writing-original draft; Investigation; Resources. LQY: methodology; writing-original draft; investigation; formal analysis. ZSY: methodology; writing-original draft; investigation; formal analysis. ZW: investigation; resources; project administration. MZR: methodology; formal analysis; conceptualization; supervision; writing-review and editing. DGT: methodology; formal analysis; conceptualization; supervision; writing-review and editing. ZXY: methodology; formal analysis; conceptualization; supervision; writing-review and editing.

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Data availability
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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